COMPOSITIONS CONTAINING AN ACTIVE FRACTION ISOLATED FROM HEDYOTIS DIFFUSAE AND METHODS OF USE

CROSS-REFERENCE TO RELATED APPLICATIONS

[001] This application claims the benefit under 35 U.S.C. § 119(e) of U.S. Provisional Application No. 60/255,502, filed December 13, 2000, the contents of which are hereby incorporated by reference into the present disclosure.

FIELD OF THE INVENTION

[002] The present invention is in the field of pharmaceuticals. In particular, it is related to the field of anti-angiogenic pharmaceuticals for the prevention and treatment of disease.

BACKGROUND

[003] Angiogenesis is the process through which new vascular structures arise by outgrowth from pre-existing capillaries. In this process, endothelial cells become detached from the basement membrane as this support is degraded by proteolytic enzymes. These cells then migrate out from the parent vessel, divide, and form into a newly differentiated vascular structure (Risau, (1997) Nature **386**:671-674; Wilting et al., (1995) Cell. Mol. Biol. Res. **41(4)**:219-232). A variety of different biological factors have been found to function in controlling blood vessel formation (Bussolino et al., (1997) Trends in Biochem Sci **22(7)**:251-256; Folkman and D'Amore, (1996) Cell **87**:1153-1155). These include proteins with diverse functions such as growth factors, cell surface receptors, proteases, protease inhibitors, and extracellular matrix proteins (Achen and Stacker, (1998) Int. J. Exp. Pathol. **79**:255-265; Devalaraja and Richmond, (1999) Trends in Pharmacol. Sci. **20(4)**:151-156; Hanahan, (1997) Science **277**:48-50; Maisonpierre et al, (1997) Science **277**:55-60; Suri et al, (1996) Cell **87**:1171-1180; Sato et al, (1995) Nature **376**:70-74; Mignatti and Rifkin, (1996) Enzyme Protein **49**:117-137; Pintucci et al., (1996) Semin Thromb Hemost **22**(6)517-524; Vernon and Sage, (1995) Am. J. Pathol. **147(4)**:873-883; Brooks et al., (1994) Science **264**:569-571; Koch et al., (1995) Nature **376**:517-519). The complexity of the angiogenic process and the diversity of the factors that control its progression provide a useful array of points for therapeutic intervention to control vascular formation *in vivo*.

[004] Angiogenesis normally occurs in a carefully controlled manner during embryonic development, during growth, and in special cases such as wound healing and the female reproductive cycle (Wilting and Christ, (1996) Naturwissenschaften **83**:153-164; Goodger and Rogers, (1995) Microcirculation **2**:329-343; Augustin et al., (1995) Am. J. Pathol. **147(2)**:339-351). Some of the important steps in the process of angiogenesis are: 1) growth factor (i.e. vascular endothelial growth factor, VEGF) signaling; 2) matrix metalloproteinases (MMP) and VEGF receptor interaction; 3) endothelial cell migration to site of growth factor signaling; and 4) endothelial cell tubule formation. Pathological angiogenesis plays a central role in a number of human diseases including tumor growth and metastatic cancer, diabetic retinopathy, rheumatoid arthritis, and other inflammatory diseases such as psoriasis (Folkman, (1995) Nature Med. **1(1)**:27-31; Polverini, (1995) Rheumatology **38(2)**:103-112; Healy et al., (1998) Hum. Reprod. Update **4(5)**:736-396). In these cases, progression of disease is driven by persistent unregulated angiogenesis. For example, in rheumatoid arthritis, new capillary blood vessels invade the joints and destroy the cartilage. In diabetic retinopathy, capillaries in the retina invade the vitreous, bleed and cause blindness. Significantly, tumor growth and metastisis are angiogenesis dependent. Most primary solid tumors go through a prolonged avascular state during which growth is limited to approximately 1-2 mm in diameter. Up to this size, tumor cells can obtain the necessary oxygen and nutrient supply by passive diffusion. These microscopic tumor masses can eventually switch on angiogenesis and recruit surrounding blood vessels to begin sprouting capillaries that vascularize the tumor mass, providing the potential for continuing expansion of the tumor and metastasis of malignant cells to distant locations. Although significant progress has been made in understanding the biological events that occur during pathological angiogenesis, there are presently no effective pharmaceutical compounds that are useful for controlling angiogensis *in vivo*. Thus, effective therapies capable of controlling angiogenesis have the potential to alleviate a significant number of human diseases.

[005] Traditionally, pharmaceutical compounds have been developed by screening synthetic chemical compounds for desirable pharmaceutical properties and then testing them for toxicity and effectiveness *in vivo*. Compounds selected this way frequently have toxic side effects *in vivo* and this approach has not been successful in developing effective angiogensis inhibitors for disease therapy. More recently, techniques of molecular biology have been applied to develop angiogenesis inhibitors. Protein inhibitors of angiogenesis such as angiostatin (O'Reilly et al., (1994) Cell **79(2)**:315-328) and endostatin (O'Reilly et al., (1997) Cell **88(2)**:277-285), that control vascular formation in experimental models, have been discovered. Nevertheless, such protein therapeutics are expensive to produce and have been found to be difficult to formulate and deliver in subjects. At present, protein angiogensis inhibitors have yet to be developed into therapeutic pharmaceuticals for disease patients. Thus, there exists a need for therapeutic compounds that can be safely administered to a patient and be effective at inhibiting the pathological growth of vascular endothelial cells. The present invention provides compositions and methods that are useful for this purpose and provides related advantages as well.

DISCLOSURE OF THE INVENTION

[006] This invention provides processes for extracting pharmaceutically active fractions (also termed "extract", "compound" or "drug") from from *Hedyotis Diffusae*. In one aspect, the process is extracting from a hot (about 80-90°C for about 30 minutes) tea of *Hedyotis Diffusae* a fraction, designated AHD04, isolated by column chromatography that has an optical absorbance between about 210 nm and about 250 nm. One means to obtain this fraction is by steeping an effective amount of *Hedyotis Diffusae* in an effective amount of hot water to obtain a liquid extract and then filtering the extract to obtain a clear liquid extract. This crude extract, designated EHDa can be processed for use as a food or health supplement. EHDa is lyophilized, resuspended and the pharmaceutically active fraction ADH04 is separated by chromatography.

[007] This invention provides a method for inhibiting the growth of endothelial cells by delivering to the cells a growth inhibitory amount of a fraction. This invention also provides a method of inhibiting vascularization in a tissue by delivering to the tissue an anti-vascularization amount of a fraction. Methods of treating various diseases, including cancer, are also provided herein.

BRIEF DESCRIPTION OF THE FIGURES

[008] Figure 1 depicts exemplary processes of this invention. However, it is to be understood, although not always explicitly stated that the reagents described herein are merely exemplary and that equivalents of such are well known in the art. The following are examples and equivalents thereof are within the scope of this invention:

[009] Figure 1 depicts procedures for isolating active fraction designated EHDa that is useful as food and health supplements as well as the process for isolating the pharmaceutically active fraction, AHD04. Briefly, the extraction of the crude extract from *Hedyotis Diffusae* starts with the steeping of the dry plant in hot water ranging in temperature from about 60°C to about 100°C for a time period of about 10 minutes to about 60 minutes. This liquid extract is then filtered using cheesecloth or Miracloth to remove all the physical items and extremely large molecules from the liquid extract. This extract is then centrifuged at 1000 rpm for approximately 10 min. The clear liquid supernatant is saved and then lyophilized to concentrate the active fraction that has been isolated from *Hedyotis Diffusae*. A CPAE assay is run to determine the effective concentrations of this new crude extract, EHDa, in inhibiting angiogenesis. All fractions, pellets, supernatants are assayed for anti-angiogenic activity using the CPAE assay after each step to insure that no anti-angiogenic activity is lost. Further isolation by concentration through chromatography, for example, yields the substantially purified active fraction AHD04.

[010] Figure 2 is a graph showing activity of the fractions as they are isolated from the G-25 gel filtration chromatography [column = volume 20 cm x 80 cm; run at 4°C and at a flow rate of 3.0 ml/mL water].

[011] Figure 3 is a graph showing activity of the fractions as they are isolated off the C- 18 chromatography column.

[012] Figure 4 is a graph where Y axis is % inhibition versus concentration (X axis) after second run through C-18 column.

MODES FOR CARRYING OUT THE INVENTION

[013] Throughout this disclosure, various publications, patents and published patent specifications are referenced by an identifying citation. The disclosures of these publications, patents and published patent specifications are hereby incorporated by reference into the present disclosure to more fully describe the state of the art to which this invention pertains.

[014] The practice of the present invention employs, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, organic chemistry, cell biology, biochemistry and immunology, which are within the skill of the art. Such techniques are explained fully in the literature.

Definitions

[015] As used herein, certain terms may have the following defined meanings.

[016] As used in the specification and claims, the singular form "a," "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a cell" includes a plurality of cells, including mixtures thereof.

[017] As used herein, the term "comprising" is intended to mean that the compositions and methods include the recited elements, but not excluding others. "Consisting essentially of" when used to define compositions and methods, shall mean excluding other elements of any essential significance to the combination. Thus, a composition consisting essentially of the elements as defined herein would not exclude trace contaminants from the isolation and purification method and pharmaceutically acceptable carriers, such as phosphate buffered saline, preservatives, and the like. "Consisting of" shall mean excluding more than trace elements of other ingredients and substantial method steps for administering the compositions of this invention. Embodiments defined by each of these transition terms are within the scope of this invention.

[018] All numerical designations, e.g., pH, temperature, time, concentration, and molecular weight, including ranges, are approximations which are varied (+) or (-) by increments of 0.1. It is to be understood, although not always explicitly stated that all numerical designations are preceded by the term "about". It also is to be understood, although not always explicitly stated, that the reagents described herein are merely exemplary and that equivalents of such are well known in the art.

[019] The term "isolated" means separated from constituents, cellular and otherwise, in which the compound is normally associated with in nature.

[020] A "subject" or "host" is a vertebrate, preferably an animal or mammal, more preferably a human patient. Mammals include, but are not limited to, murines, simians, human patients, farm animals, sport animals, and pets.

[021] The terms "cancer," "neoplasm," and "tumor," used interchangeably and in either the singular or plural form, refer to cells that have undergone a malignant transformation that makes them pathological to the host organism. Primary cancer cells (that is, cells obtained from near the site of malignant transformation) can be readily distinguished from non-cancerous cells by well-established techniques, particularly histological examination. The definition of a cancer cell, as used herein, includes not only a primary cancer cell, but also any cell derived from a cancer cell ancestor. This includes metastasized cancer cells, and *in vitro* cultures and cell lines derived from cancer cells. When referring to a type of cancer that normally manifests as a solid tumor, a "clinically detectable" tumor is one that is detectable on the basis of tumor mass; e.g., by such procedures as CAT scan, magnetic resonance imaging (MRI), X-ray, ultrasound or palpation. Biochemical or immunologic findings alone may be insufficient to meet this definition.

[022] As used herein, "inhibit" means to stop, delay or slow the growth, proliferation or cell division of endothelial cells or the formation of blood vessels in tissue. Methods to monitor inhibition include, but are not limited to endothelial cell proliferation assays, measurement of the volume of a vascular bed by determination of blood content and quantitative determination of the density of vascular structures. When the culture is a mixture of cells, neovascularization is monitored by quantitative measurement of cells expressing endothelial cell specific markers such as angiogenic factors, proteolytic enzymes and endothelial cell specific cell adhesion molecules.

[023] A "composition" is intended to mean a combination of active agent and another compound or composition, inert (for example, a detectable agent or label) or active, such as an adjuvant.

[024] A "pharmaceutical composition" is intended to include the combination of an active agent with a carrier, inert or active, making the composition suitable for diagnostic or therapeutic use *in vitro, in vivo or ex vivo*.

[025] As used herein, the term "pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutical carriers, such as a phosphate buffered saline solution, water, and emulsions, such as an oil/water or water/oil emulsion, and various types of wetting agents. The compositions also can include stabilizers and preservatives. For examples of carriers, stabilizers and adjuvants, see Martin, REMINGTON'S PHARM. SCI., 15th Ed. (Mack Publ. Co., Easton (1975)).

[026] An "effective amount" is an amount sufficient to effect beneficial or desired results. For example, a therapeutic amount is one that achieves the desired therapeutic effect. This amount may be the same or different from a prophylatically effective amount, which is an amount necessary to prevent onset of disease or disease symptoms. An effective amount can be administered in one or more administrations, applications or dosages.

[027] Applicant has identified a process for extracting pharmaceutically active fractions from *Hedyotis Diffusae*. In one aspect, the process requires extracting from a hot (about 80-90°C for about 30 minutes) tea of *Hedyotis Diffusae* a fraction wherein said fraction has an optical absorbance between about 210 nm and about 250 nm. In a further aspect, the active fraction has an optical absorbance of about 220 to about 240 nm. In a yet further aspect, the pharmaceutically active fraction has an optical absorbance after chromatography of about 230 nm. An effective amount of *Hedyotis Diffusae* is steeped in an effective amount of hot water to obtain a liquid extract and then filtering the extract to obtain a clear liquid extract. The extracted is lyophilized and then resuspended in a suitable carrier and the pharmaceutically active fraction is separated by chromatography.

[028] The inventor has also discovered that the fractions inhibit endothelial cell growth and possess anti-angiogenic properties. In accordance with these findings, this invention provides a method for inhibiting the growth of endothelial cells by delivering to the cells a growth inhibitory amount of a fraction. This invention also provides a method of inhibiting vascularization in a tissue by delivering to the tissue an anti-vascularization amount of the fraction.

[029] This method can be practiced *in vitro* or *in vivo*. When practiced *in vitro*, endothelial cells or vascularized tissue are cultured under conditions well known to those of skill in the art, e.g., as exemplified below. The cells and/or tissue can be from an established cell line or cultured from a biopsy sample obtained from a subject. The fraction is then directly added to the culture medium or delivered as a component of a pharmaceutical composition.

[030] Not every therapy is effective for each individual and therefore, an *in vitro* assay to gauge efficacy for each patient would be advantageous. The present method provides these means to determine whether the methods of this invention will treat the patient. For example, a tissue biopsy is isolated from the patient and contacted with an effective amount of a pharmaceutical composition or therapy as defined herein and under conditions effective for growth and proliferation of the cells. Inhibition of growth of the pathological cells as determined by conventional procedures, e.g., the CPAE assay described herein, indicates that the inventive compositions and/or therapies may effectively treat the patient.

[031] Angiogenesis or the formation of new vasculature is a fundamental process by which new blood vessels are formed. It participates in essential physiological events, such as reproduction development and wound healing. Under normal conditions, angiogenesis is highly regulated. However, many diseases are driven by persistent unregulated angiogenesis. In rheumatoid arthritis, new capillary blood vessels invade the joints and destroy the cartilage. In diabetic retinopathy, new capillaries in the retina invade the vitreous, bleed, and cause blindness. Tumor growth and metastasis are angiogenesis-dependent. Most primary solid tumors go through a prolonged state of avascular, and apparently dormant, growth in which the maximum size attainable is ~1-2mm in diameter. Up to this size, tumor cells can obtain the necessary oxygen and nutrient by simple passive diffusion. These microscopic tumor masses can eventually switch on angiogenesis by recruiting surrounding mature host blood vessels to begin sprouting new blood vessel capillaries which grow toward, and eventually infiltrate the tumor mass, thus setting in motion the potential for relentless expansion of tumor mass and hematogenous metastatic spread as well. The angiogenic switch was initially hypothesized to be triggered by the ectopic production and elaboration by tumor cells of a growth factor called "tumor angiogenesis factor" (TAF).

[032] This invention also provides a method of treating a disorder associated with pathological neovascularization in a subject by administering to the subject a therapeutically effective amount of the extract or a pharmaceutical composition containing the extract. As used in this context, to "treat" means to alleviate the symptoms associated with pathological neovascularization as well as the reduction of neovascularization. Such conditions include, but are not limited to arthritic conditions, neovascular-based dermatological conditions, diabetic retinopathy, Karposi's Sarcoma, age-related macular degeneration, restenosis, telangectasia, glaucoma, keloids, comeal graft rejection, wound granularization, angiofibroma, Osler-Webber Syndrome, myocardial angiogenesis, and scleroderma. Exemplary arthritic conditions are selected from the group consisting of rheumatoid arthritis, psoriatic arthritis and osteoarthritis. For the treatment of cancers and solid tumors, to "treat" includes inhibition of the growth of blood vessels resulting in a lack of nutrients for the tumors and/or cancer cells needed by the tumor for its growth. Tumors and growths will decrease in size and possibly disappear. Administration for the treatment of arthritic conditions will result in decreased blood vessel formation in cartilage, specifically joints, resulting in increased mobility and flexibility in these regions. For the treatment of psoriasis, administration will reduce dermatological symptoms such as scabbing, flaking and visible blood vessels under the surface of the skin. In diabetic retinopathy, administration of the active fraction will reduce the formation of extraneous blood vessels in the retina, resulting in unobstructed vision. In the treatment of Kaposi's Sarcoma, administration of the active fraction will inhibit the growth and/or further formation of blood vessels, thereby inhibiting the formation of lesions and/or tumors that arise.

[033] When the active fraction is administered to a subject such as a mouse, a rat or a human patient, the agent can be added to a pharmaceutically acceptable carrier and systemically, orally, transdermally or topically administered to the subject. Therapeutic amounts can be empirically determined and will vary with the pathology being treated, the subject being treated and the toxicity of the form of the fraction used in the therapeutic method. The active fraction can be delivered orally, intravenously, intraperitoneally, or transdermally. When delivered to an animal, the method is useful to further confirm efficacy of the active fraction.

[034] As an example of an animal model, groups of nude mice (Balb/c NCR nu/nu female, Simonsen, Gilroy, CA) are each subcutaneously inoculated with about 10^5 to about 10^9 pathological cells as defined herein. When the graft is established, the fraction, extract or compound is administered, for example, by subcutaneous injection around the graft. Measurements to determine reduction of graft size are made in two dimensions using venier calipers twice a week.

[035] The MRL/lpr mice (MRL/MpJ-Fas^{lpr}) from Jackson Labs (Maine) are useful to test or monitor efficacy in arthritic conditions. A positive therapeutic benefit includes reduced swelling of the joints and hindlegs of animals and reduced cartilage degradation which can be monitored by X-ray.

[036] Administration *in vivo* can be effected in one dose, multiple doses, continuously or intermittently throughout the course of treatment. Methods of determining the most effective means and dosage of administration are well known to those of skill in the art and will vary with the composition used for therapy, the purpose of the therapy, the target cell being treated, and the subject being treated. Single or multiple administrations can be carried out with the dose level and pattern being selected by the treating physician. Suitable dosage formulations and methods of administering the agents can be found below.

[037] The compositions and pharmaceutical formulations of the present invention can be used in the manufacture of medicaments and for the treatment of humans and other animals by administration in accordance with conventional procedures, such as an active ingredient in pharmaceutical compositions.

[038] The pharmaceutical compositions can be administered orally, intranasally, parenterally or by inhalation therapy, and may take the form of tablets, lozenges, granules, capsules, pills, ampoules, suppositories or aerosol form. They may also take the form of suspensions, solutions and emulsions of the active ingredient in aqueous or nonaqueous diluents, syrups, granulates or powders. In addition to a compound of the present invention, the pharmaceutical compositions can also contain other pharmaceutically active ingredients.

[039] More particularly, the active fraction also referred to herein as the active ingredient, may be administered for therapy by any suitable route including oral, rectal, nasal, topical (including transdermal, aerosol, buccal and sublingual), vaginal, parental (including subcutaneous, intramuscular, intravenous and intradermal) and pulmonary. It will also be appreciated that the preferred route will vary with the condition and age of the recipient, and the disease being treated.

[040] It will be appreciated that appropriate dosages of the active fraction of the invention may depend on the type and severity and stage of the disease and can vary from patient to patient. Determining the optimal dosage will generally involve the balancing of the level of therapeutic benefit against any risk or deleterious side effects of the treatments of the present invention.

[041] Ideally, the fraction, extract, or compound ("drug") or composition containing it should be administered to achieve peak concentrations of the active compound at sites of disease. This may be achieved, for example, by the intravenous injection of the drug, optionally in saline, or orally administered, for example, as a tablet, capsule or syrup containing the active ingredient. Desirable blood levels of the drug may be maintained by a continuous infusion to provide a therapeutic amount of the active ingredient within disease tissue. The use of operative combinations is contemplated to provide therapeutic combinations requiring a lower total dosage of each component agent than may be required when each individual therapeutic compound or drug is used alone, thereby reducing adverse side effects.

[042] While it is possible for the drug ingredient to be administered alone, it is preferable to present it as a pharmaceutical formulation comprising at least one active ingredient, as defined above, together with one or more pharmaceutically acceptable carriers therefor and optionally other therapeutic agents. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the patient.

[043] Formulations include those suitable for oral, rectal, nasal, topical (including transdermal, buccal and sublingual), vaginal, parenteral (including subcutaneous, intramuscular, intravenous and intradermal) and pulmonary administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. Such methods include the step of bringing into association the active ingredient with the carrier that constitutes one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing into association the active ingredient with liquid carriers or finely divided solid carriers or both, and then if necessary shaping the product.

[044] Formulations of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets or tablets, each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or suspension in an aqueous or non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion. The active ingredient may also be presented a bolus, electuary or paste.

[045] A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredient in a free-flowing form such as a powder or granules, optionally mixed with a binder (e.g., povidone, gelatin, hydroxypropylmethyl cellulose), lubricant, inert diluent, preservative, disintegrant (e.g., sodium starch glycolate, cross-linked povidone, cross-linked sodium carboxymethyl cellulose) surface-active or dispersing agent. Molded tablets may be made by molding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets may optionally be coated or scored and may be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropylmethyl cellulose in varying proportions to provide the desired release profile. Tablets may optionally be provided with an enteric coating, to provide release in parts of the gut other than the stomach.

[046] Formulations suitable for topical administration in the mouth include lozenges comprising the active ingredient in a flavored basis, usually sucrose and acacia or tragacanth; pastilles comprising the active ingredient in an inert basis such as gelatin and glycerin, or sucrose and acacia; and mouthwashes comprising the active ingredient in a suitable liquid carrier.

[047] Pharmaceutical compositions for topical administration according to the present invention may be formulated as an ointment, cream, suspension, lotion, powder, solution, paste, gel, spray, aerosol or oil. Alternatively, a formulation may comprise a patch or a dressing such as a bandage or adhesive plaster impregnated with active ingredients and optionally one or more excipients or diluents.

[048] For diseases of the eye or other external tissues, e.g., mouth and skin, the formulations are preferably applied as a topical ointment or cream containing the active ingredient. When formulated in an ointment, the drug may be employed with either a paraffinic or a water-miscible ointment base. Alternatively, the drug ingredients may be formulated in a cream with an oil-in-water cream base.

[049] If desired, the aqueous phase of the cream base may include, for example, at least about 30% w/w of a polyhydric alcohol, i.e., an alcohol having two or more hydroxyl groups such as propylene glycol, butane-1,3-diol, mannitol, sorbitol, glycerol and polyethylene glycol and mixtures thereof. The topical formulations may desirably include a compound that enhances absorption or penetration of the drug ingredient through the skin or other affected areas. Examples of such dermal penetration enhancers include dimethylsulfoxide and related analogues.

[050] The oily phase of the emulsions of this invention may be constituted from known ingredients in any known manner. While this phase may comprise merely an emulsifier (otherwise known as an emulgent) it desirably comprises a mixture of at lease one emulsifier with fat or oil or with fat and oil. Preferably, a hydrophilic emulsifier is included together with a lipophilic emulsifier that acts as a stabilizer. It is also preferred to include both oil and fat. Together, the emulsifier(s) with or without stabilizer(s) make up the so-called emulsifying wax, and the wax together with the oil and/or fat make up the so-called emulsifying ointment base which forms the oily dispersed phase of the cream formulations.

[051] Emulgents and emulsion stabilizers suitable for use in the formulation of the present invention include Tween 60, Span 80, cetostearyl alcohol, myristyl alcohol, glyceryl monostearate and sodium lauryl sulphate.

[052] The choice of suitable oils or fats for the formulation is based on achieving the desired cosmetic properties, since the solubility of the active compound in most oils likely to be used in pharmaceutical emulsion formulations is very low. Thus the cream should preferably be a non-greasy, non-staining and washable product with suitable consistency to avoid leakage from tubes or other containers. Straight or branched chain, mono- or dibasic alkyl esters such as di-isoadipate, isocetyl stearate, propylene glycol diester of coconut fatty acids, isopropyl myristate, decyl oleate, isopropyl palmitate, butyl stearate, 2-ethylhexyl palmitate or a blend of branched chain esters known as Crodamol CAP may be used. These may be used alone or in combination depending on the properties required. Alternatively, high melting point lipids such as white soft paraffin and/or liquid paraffin or other mineral oils can be used.

[053] Formulations suitable for topical administration to the eye also include eye drops wherein the active ingredient is dissolved or suspended in a suitable carrier, especially an aqueous solvent for the active ingredient. Formulations for rectal administration may be presented as a suppository with a suitable base comprising, for example, cocoa butter or a salicylate.

[054] Formulations suitable for vaginal administration may be presented as pessaries, tampons, creams, gels, pastes, foams or spray formulations containing in addition to the active ingredient, such carriers as are known in the art to be appropriate.

[055] Formulations suitable for nasal administration, wherein the carrier is a solid, include a coarse powder having a particle size, for example, in the range of about 20 to about 500 microns which is administered in the manner in which snuff is taken, i.e., by rapid inhalation through the nasal passage from a container of the powder held close up to the nose. Suitable formulations wherein the carrier is a liquid for administration as, for example, nasal spray, nasal drops, or by aerosol administration by nebulizer, include aqueous or oily solutions of the active ingredient.

[056] Formulations suitable for parenteral administration include aqueous and nonaqueous isotonic sterile injection solutions which may contain anti-oxidants, buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents, and liposomes or other microparticulate systems which are designed to target the compound to blood components or one or more organs. The formulations may be presented in unit-dose or multi-dose sealed containers, for example, ampoules and vials, and may be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

[057] Preferred unit dosage formulations are those containing a daily dose or unit, daily subdose, as herein above recited, or an appropriate fraction thereof, of a drug ingredient.

[058] Preferred unit dosage formulations are those containing a daily dose or unit, daily subdose, as herein above recited, or an appropriate fraction thereof, of a drug ingredient. They may also contain additional active agents, e.g., anti-tumor, anti-cancer, anti-angiogenic or immune enhancers.

[059] It should be understood that in addition to the ingredients particularly mentioned above, the formulations of this invention may include other agents conventional in the art having regard to the type of formulation in question, for example, those suitable of oral administration may include such further agents as sweeteners, thickeners and flavoring agents.

[060] The extract ("drug") or compositions of the same may also be presented for the use in the form of veterinary formulations, which may be prepared, for example, by methods that are conventional in the art.

[061] This invention further provides a method for screening for a therapeutic agent for inhibiting neovascularization or endothelial cell growth. The screen comprises:

(a) contacting the agent with a suitable cell or tissue sample;

(b) contacting a separate sample of the suitable cell or tissue with a therapeutically effective amount of an extract of this invention or a pharmaceutically acceptable composition containing the extract; and

(c) comparing the growth of the sample of step (a) with the growth of the sample of step (b), and wherein any agent of step (a) that inhibits the growth to the same or similar extent as the sample of step (b) is a therapeutic agent for inhibiting neovascularization or the growth of endothelial cells.

[062] As used herein, a suitable sample intends any sample that contains endothelial cells. The method can be practiced *in vitro* or *in vivo* as described herein.

[063] A kit for treating a disorder associated with pathological neovascularization in a subject, also is provided by this invention. The kit includes a therapeutically effective amount of an extract and instructions for use. The kit is useful to treat disorders selected from the group consisting of arthritic conditions, neovascular-based dermatological conditions, diabetic retinopathy, Karposi's Sarcoma, age-related macular degeneration, restenosis, telangectasia, glaucoma, keloids, comeal graft rejection, wound granularization, angiofibroma, Osler-Webber Syndrome, myocardial angiogenesis, scleroderma, rheumatoid arthritis, psoriatic arthritis and osteoarthritis.

[064] The following examples are intended to illustrate, but not limit the invention.

[065] **EXAMPLES**

Materials:

The following materials were used in the methods described below.

[066] The dry plant of Herba *Hedyotis Diffusae* was homogenized and extracted by hot water (80°-90°C (Sigma); sodium acetate (Sigma); ammonium sulfate (Sigma); hydrochloric acid (VWR Scientific); phosphatase substrate (Sigma); Sephadex G-25 (Sigma); and Triton X- 100 (Sigma).

[067] Example 1

Isolation and Purification

This invention provides several embodiments of a process for preparing biologically active fractions from *Hedyotis Diffusae*. In one aspect, the process, shown for example in Figure 1, comprises extracting a soluble fraction AHD04 that has an optical absorbance of between about 210 nm to about 250 nm. In a further aspect, the fraction has an absorbance from about 220 nm to and 240 nm. In a yet further aspect, the active fraction's absorbance is about 230 nm. In a further aspect, leaves and/or stems of the plant are steeped in hot water (any temperature at or above about 60°C, and preferably above about 90°C). The tea is extracted and concentrated to provide EHDa.

[068] Example 2

Determination of Angiogenesis-Inhibition by Endothelial Cell Culture (EGG) Assay

The assay used to determine the percentage of angiogenesis inhibition was a variation of the assay developed by D.T. Connally, et al. (1986) Anal. Biochem. **152**:136-4 with modifications (Liang and Wong (1999) ANGIOGENESIS: FROM THE MOLECULAR TO INTEGRATIVE PHARMACOLOGY edited by Maradoudakis, Kluwer Academic/Plenum. Publishers, New York) for the determination of cell number by the level of acid phosphatase activity. CPAE (Cardiopulmonary Artery Endothelial Cells, bovine) acquired from American Type Tissue Culture (ATTC) were grown to nearly 95% confluence in MEM-10E. The cells were released from the tissue culture flask with a 0.25% trypsin solution and planted in 24-well tissue culture plates in the same culture medium at a density of 10,000 cell/well. After the plates were cultivated for 8 hours at 37°C in a 5.0% CO_2 incubator. Assay samples and controls were added. Each sample was loaded in two different wells at 100 μL/well to insure reproducibility. After incubation with the sample for 60 hours, the medium was aspirated, and the number of cells was measured on the basis of the colorimetric measurement of cellular acid phosphatase.

[069] **Example 3**

Cytolytic/Cytotoxic Assay

Calf Pulmonary Arterial Endothelial (CPAE) cells are plated at 10,000 cells per well in 24 well culture plates. After growth incubation at 37°C, 5% CO_2 for about 60 hours, a dosage of the sample is added (about 50µl to about 100µl) to each sample well and re-incubated for 30 minutes. After incubation, cells are assayed visually under an inverted microscope to detect the presence of cells and through the use of the ECC assay. Both methods are used to detect the presence or absence of endothelial cells in each well. Control cells containing no sample were used and grew normally.

[070] **Example 4**

Sample Titration Assay

To determine if anti-aniogenesis activity from a fraction, e.g., ADH04 is dosage related, the titration assay was carried out on endothelial cells. Sample was made in concentration titrations from 1.0mg/mL to 0.00625mg/mL. Different samples were loaded on cell, while blank is the control. Results are shown in Table 1 (below).

Table 1: Angiogenesis Inhibition Assay of AHD04 at Different Concentration

Concentration (µg/mL)	100	50	25	2.5
o/o Inhibition	83	75	0	0

[071] **Example 5**

Inhibition Specificity

In order to determine whether this inhibition activity is specific to endothelial cells, FCC assay was carried out by using HEP-2 cells (isolated from human laryngeal tissue) as control. The results show that HEP-2 cells are not sensitive to the active fraction.

[072] The results are shown in Table 2.

Table 2

Concentration (μg/mL)	% CPEA Cell Inhibited	% HEP-2 Cell Inhibited
100	86	0
50	84	0
10	30	0
5	0	0

[073] **Example 6**

MMP Assay

P.C. Brooks, et. al. (1996) in "Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin αvβ3," Cell **85**:683-93 describes an *in vitro* assay on matrix metalloproteinase and αv/β3 integrin interaction. The effects of the experimental sample on the MMP-2/αvβ3 integrin complex determines if the sample's mechanism of action involves any disruption of this segment of the angiogenic pathway. This involves testing if the experimental sample can inhibit the interaction of MMP-2 with the αvβ3 integrin. Initially, this is done via an ELISA using antibodies for MMP-2 and testing the binding of these antibodies to the sample. Further studies are pursued if a positive result occurs. TIMP-2 (Tissue Inhibitor of Matrix Metalloprotease-2), a known natural inhibitor of MMP-2, is used as the control.

[074] **Example 7**

Endothelial Cell Tubule/Cord Formation Assay

Matrigel (60µl of 10mg/ml; Collaborative Lab # 35423) is placed in each well of an ice-cold 96-well plate. The plate is allowed to sit at room temperature for 15 minutes then incubated at 37^0C for 30 minutes to permit the matrigel to polymerize. In the mean time, HUVEC are prepared in EGM-2 (Clonetic # CC3162) at a concentration of $2X10^5$ cells/ml. The test compound is prepared at 2X the desired concentration (5 concentration levels) in the same medium. Cells (500µl) and 2X fraction or compound (500µl) is mixed and 200µl of this suspension are placed in duplicate on the polymerized matrigel. After a 24 hour incubation, triplicate pictures are taken for each concentration using a Bioquant Image

Analysis system. Drug effect (IC_{50}) is assessed compared to untreated controls by measuring the length of cords/tubules formed and number of junctions. TNP-470 (NSC 642492) and paclitaxel (NSC 125973) are used as reference compounds.

[075] Example 8

Endothelial Cell Migration Assay

Migration is assessed using the 48-well Boyden chamber and 8μm pore size collagen-coated (10μg/ml rat tail collagen; Collaborative Laboratories) polycarbonate filters (Osmonics, Inc.). The bottom chamber wells receive 27-29μl of DMEM medium alone (baseline) or medium containing chemo-attractant (bFGF, VEGF or Swiss 3T3 cell conditioned medium). The top chambers receive 45μl of HUVEC cell suspension ($1X10^6$ cells/ml) prepared in DMEM+1% BSA with or without the fraction or compound. After a 5 hour incubation at 37^0C, the membrane is rinsed in PBS, fixed and stained in Diff-Quick solutions. The filter is placed on a glass slide with the migrated cells facing down and cells on top are removed using a Kimwipe. The testing is performed in 4-6 replicates and five fields are counted from each well. Negative unstimulated control values are subtracted from stimulated control and fraction or compound treated values and data is plotted as mean migrated cell ± S.D. IC_{50} is calculated from the plotted data. TNP-470 (NSC 642492) and paclitaxel (NSC 125973) are used as reference compounds.

[076] Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be apparent to those skilled in the art that certain changes and modifications will be practiced. For example, as is apparent to those of skill in the art, the invention method can be combined with one or more known anti-tumor, anti-angiogenic or immune enhancing therapies and compositions, e.g., shark cartilage, tyrosphingosine or sphingosine. Therefore, the description and examples should not be construed as limiting the scope of the invention, which is delineated by the appended claims.